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In Vivo Acute Toxicity, Anticancer And Antioxidant Assay Evaluation Of Thunbergia Grandiflora.

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ABSTRACT
Thunbergia grandiflora, commonly called blue trumpet vine, sky vine or sky flower belongs to Acanthaceae, which is native in Australia, Central America, the West Indies, Africa, and numerous islands in the Pacific including Hawaii, Fiji, French Polynesia, Palau, and Samoa.
In the present investigation, Thunbergia Grandiflora extracts were studied for in-vivo acute toxicity, anticancer and antioxidant activity. According to the toxicity study, 200 and 400mg/kg body weight was selected as dose. In the evaluation of anticancer activity the tumour yield and tumour burden were not detected up to 4th weeks; but in 8th week, observed the formation of tumours and it become maximum in 16th week in all treated groups of mice. In carcinogenic control group of mice maximum tumors activity was observed due to carcinogenic nature but it got decreased after the treatment with sample 1,2,3 and isolated compound. Also, carcinogen increased LPO and reduced SOD, GSH, and catalase in lung homogenate. Extracts significantly reversed the effect of carcinogen on LPO level. The isolated compound was found more effective than the extracts (200 mg/kg an 400 mg/kg) on LPO, SOD, GSH and catalase. Moreover the extract of Thunbergia grandiflora showed down regulated effect on the levels of TNF-α and IL-6.

KEYWORDS
Thunbergia grandiflora, Acute Toxicity, Anticancer, Antioxidant Assay In-Vivo.

1. INTRODUCTION

Medicinal plants are used as a means of disease relief that can be traced back over five millennia to long-term written documents of the early civilizations of India. Plants have become a reliable source of natural products for the protection of human health, with more comprehensive natural therapy studies, especially in the last decade. The plant kingdom is a mostly unexplored reservoir of biologically active compounds, not only as drugs, but also as basic models that may serve as a starting point for synthetic analogues and as an interesting method that can be used to better understand biological processes. The use of plant compounds has slowly increased in India for medicinal purposes. Medicinal plants can be the safest source for a range of drugs, according to the World Health Organization. Around 80 percent of individuals from developed countries use herbal medicine, which has compounds derived from medicinal plants.

Plants have been found to be a very viable source of anticancer compounds that are clinically significant. Ethnopharmacological information in the quest for new cancer concepts has however been misused in the past. In many ethnomedical systems, records of particular plant antitumor uses are seldom seen, largely because cancer is a disorder involving a complex range of signs and symptoms. As many plant products and their derivatives are approved for the control of cancer, it is highly beneficial to produce new medicines to play an important role in the control of cancer.

To define the different causes of cancer, several technical terms have been used. Sarcoma is a cancer of the connective tissues of the body, including bone, skin, cartilage, and blood vessels, and bone marrow cancer is leukaemia. Myeloma, such as carcinoma, is an immune system disease. By far, lung cancer is the most common malignant lung tumour. More than 30 percent of lung cancers are histologically distinct. Clearly, through its histology, neuroendocrine heterogeneity, transient chemotherapy response and, usually poor survival, small cell lung cancer can be distinguished from non-small cell lung cancer (NSCLCs). [7] The

Cancer is responsible for potential DNA and epigenetic changes that as well as various risk factors, will lead to smoking, radon gas, asbestos, air pollution and genetics.

Therapy for NSCLC focuses on cancer stages. Step I, Stage II, Stage III and Stage IV, Chemotherapy, Fourth-Phase Chemotherapy, Guided Fourth-Line Treatment, First-Phase EGFR Tyrosine Kinase, First-Line Reconstruction of Chemotherapy, Second-Line and Third-Line Advanced NSCLC Therapy, Chronic NSCLC Cytototoxic Chemotherapy and NSCLC Therapy, Chronic EGFR NSC Inhibitors. [1-5] The

For the study, Thunbergia Grandiflora was selected based on data available from folk studies and recent experimental studies. As an ornamental garden and a wall, it is commonly cultivated in Kenya.

The anticancer, antioxidant and toxicity profile analysis of Thunbergia grandiflora using in-vivo models is the subject of current research.

2. MATERIALS AND METHODS

Materials

Authentication of Plant

The plant was identified and authenticated by Dr. Saba Khan, Botanist, Safia college of Science, Bhopal. A voucher specimen number 132/Bot/Safia/19 was kept in Department of Botany, Safia college of Science, Bhopal for future reference.

Chemicals

All the chemicals used were of analytical grade and were obtained from Merck or Sigma or S.D. Fine Chemicals.

Methods [7-17]

Solvent Extraction

Cold maceration

The leaves of Thunbergia grandiflora were picked, washed thoroughly and rinsed. They were dried and chemically powdered in the shade. Approximately 500gm of powder was macerated with Pet's leaf. Ether, ethyl acetate, and methanol cause phytochemicals to stand for 4-5 days each under ice cold conditions for extraction. Both non-extractable products, including cellular materials and other constitutions that are insoluble in the extraction solvent, were filtered with Whatmann No. 1 Philtre paper at the end of the third day. The entire extract was condensed into dryness using a rotary flash evaporator under decreased pressure

and placed in an airtight container free of all contamination once used. The dried extract percentage yields were eventually determined.

Extraction yield (%) = (weight of the dried extract)/ (weight of original plant sample) ×100. ---Formula 1

In-vivo acute toxicity study (OECD 423 guidelines)
Acute oral toxicity study
As per the recommendation for the OECD dose collection 423. A stepwise protocol requiring the use of 3 single-sex animals per phase is the acute toxic class approach set out in this Guideline. The determination of the acute toxicity of the test item, based on the mortality and/or the moribund condition of the animals, can entail an average of 4 steps. The medication is given orally to a group of laboratory animals at one of the specified doses. To test the stuff, a step-by-step procedure is used, each step using three animals of a single sex (normally females). No more study, dosing of three additional animals at the same dosage, and dosing of three additional animals at the next higher or subsequent lower dose level could be needed for the absence or presence of compound-related mortality of the animals dosed at a single stage. Three animals are used for each point. A dosage level to be used as the starting dose is selected from one of the four set ranges, 5, 50, 300 and 2000 mg/kg of body weight.

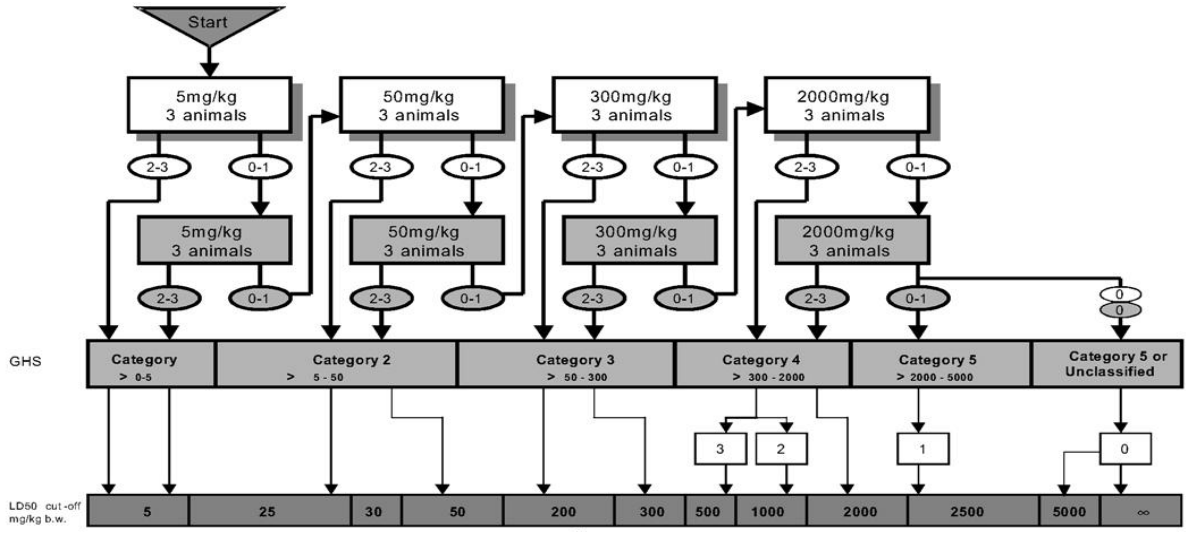


Figure 1. Flowchart for acute oral toxicity study (Annexure 2a, OECD 423 guidelines).

In vivo Anti-cancer activity (Lung cancer)[18,20-23]
Wistar rats were divided into management and drug therapy groups of at least six animals each. BaP-induced lung cancer-bearing rats (BaP-induced lung cancer-bearing rats): Animals were fed orally twice a week for four weeks, with a regular diet and water supplementation. BaP was prepared by mixing with 1 ml of olive oil and a 50 mg/kg body weight dose was given to each rat for four weeks, twice a week using gavage. A daily diet and TG research drugs were fed to rats until the end of the experiment (16 weeks) after one month of scheduled BaP feeding. During the investigative era, the animals were slaughtered. The tumour's body weight and volume have been determined. In a Tris-HCl buffer (0.1 M, pH 7.4), lung tissue was well washed with ice-cold saline and homogenised for biochemical assay.

Table 1. Treatment given to different groups.

S. No	Group	Treatment	Total No of animals required
1	Group 1	Cancer was induced when animals received BaP orally twice a week for four weeks, followed by a normal diet and water.	06
2	Group 2	After one month of scheduled BaP feeding, rats were treated orally with test drug TG 200 mg.kg bw daily for eight weeks.	06
3	Group 3	After one month of scheduled BaP feeding, rats were treated orally with test drug TG 400 mg.kg bw daily for eight weeks.	06
4	Group 4	After one month of scheduled BaP feeding, rats were treated orally with isolated drug sample for eight weeks.	06

In Vivo Antioxidant Assay[19, 24-30]
In vivo Biochemical Assay for enzymes involved in oxidative stress
I. Dissection and homogenization
After the research was ended, the specimens were sacrificed shortly after behavioural examination by decapitation. The lungs were cut, dissected, rinsed, and measured with isotonic saline.
II. Preparing tissue for assay
The lung was rinsed with ice cold normal saline followed by 0.15 M tris HCl (pH 7.4) then following procedure was followed for particular assay.
A] Lipid peroxidase (LPO)
10 % w / v tissue homogenate was prepared in a 0.15 M tris HCl buffer (pH 7.4). 8.1 percent SDS + 1.5 ml 20 percent acetic acid + 1.5 ml 8 percent TBA was combined and filtered water was applied to make up to 4 ml of volume. 0.2 ml tissue homogenate + 0.2 ml Using a glass ball as a condenser, the reaction blend was heated for 60 minutes in a water bath (95 °C). A volume of up to 5 ml was produced and the mixture was cooled. 5 ml butanol: Pyridine (15:1) has been added. It was then vortexed after that for 2 minutes and centrifuged at 3000 rpm for 10 minutes. The upper organic layer was obtained and examined as a blank with a spectrophotometer at 532 nm using butanol:pyridine / 15:1. The basic theory is that TBA interacts with MDA to form pink TBARS in an acidic medium. In total, the absorbance was made of MDA. The perception would rely on the standard curve of the MDA.

B] Superoxide dismutase (SOD)
Procedure
10 percent w/v tissue homogenate was prepared and centrifuged at 15000 rpm for 15 min at 4 ° C at 0.15 M Tris HCl or 0.1 M phosphate buffer. 0.1 ml of supernatant was taken, and is known as a study. 0.1 ml sample + 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052 M) + 0.1 ml phenazine methosulphate buffer (186 μM) + 0.3 ml 300 μM Nitroblutetrazolium + 0.2 ml NADH (750 μM) buffer. At 30°C, the mixture was incubated for 90 seconds. 0.1 ml of glacial acetic acid and 4.0 ml of n-butanol were added and combined. The mixture was permitted to stand and then for 10 min, centrifuged. A sheet of butanol whose OD has been tested at 560 nm is isolated. It was understood that Butanol was blank.

On the basis of the SOD concentration curve percentage inhibition, interpretation is carried out.

$$\% \text{ Inhibition} = \frac{(\Delta \text{ Absorbance}_{\text{control}} - \Delta \text{ Absorbance}_{\text{sample}}) \times 100}{\Delta \text{ Absorbance}_{\text{control}}}$$

---Formula 1---

*Control consists of all reaction mixture except sample, instead of sample equal amount of buffer/water was taken.

C] Glutathione (GSH)

10 percent w/v tissue homogenate was prepared in phosphate buffer 0,1 M, pH 7,4. It was taken with 0.2 ml homogenate; 20 percent TCA and 1 mM EDTA were added to it. It was stored for 5 minutes, then centrifuged at 2000 rpm for 10 minutes. We took 200 µl of supernatant and transferred it to another tube. Added 1.8 ml of Ellman's reagent (5,5'-dithium bis-2-nitrobenzoic acid (0.1mM) prepared with 1% sodium citrate solution in a 0.3 M phosphate buffer pH 7) [stored in the dark at 0-5°C]. Up to 2 ml was the volume of purified water. At 412 nm, OD was measured using blank water using a spectrophotometer.

D] Catalase

Procedure

2.9 ml of H_2O_2 solution was taken and observed at 240 nm till absorbance becomes stable. 2.9 ml of H_2O_2 solution and 0.1 ml test sample was added. It mix by inversion and the time required for the A240nm to decrease from 0.45 to 0.40 absorbance units was recorded.

ELISA based Estimation

1. Estimation of Tumour Necrosis Factor – Alpha (TNF-α)

For estimation of TNF-α 'The RayBio® Rat/Mice TNF-alpha ELISA (Enzyme-Linked Immunosorbent Assay) kit' was used.

Preparation of standard

Item C vial Standard Recombinant Rat/Mice TNF-alpha was spinned to shake the stuff and 400 µl Assay Diluent A was added to serum/plasma samples) to prepare a standard of 100 ng/ml. The powder was carefully mixed and 200µl of this solution to prepare a standard stock solution (20,000 pg/ml) was taken into a tube of 400µl of Assay Diluent A. Afterwards with 400 µl of Assay Diluent A, 100 µl of this solution was blended into each tube. Using the stock standard solution, a dilution sequence (shown in fig 3.1) was created. Each tube was combined thoroughly prior to the next step. The assay of Diluent A served as the zero norm (0 pg/ml).

Preparation of wash buffer

20 ml of Wash Buffer Concentrate (20x) (Item B) was diluted with deionized or distilled water to yield 400 ml of 1x Wash Buffer solution.

Preparation of Detection Antibody vial (Item F)

The content of detection antibody vial (item F) was proportionately added with 1x Assay Diluent A until the Detection Antibody concentration diluted to 80-fold.

Preparation of HRP-Streptavidin concentrate vial (Item G)

HRP - Streptavidin concentrate was diluted to 10,000-fold with 1x Assay Diluent A.

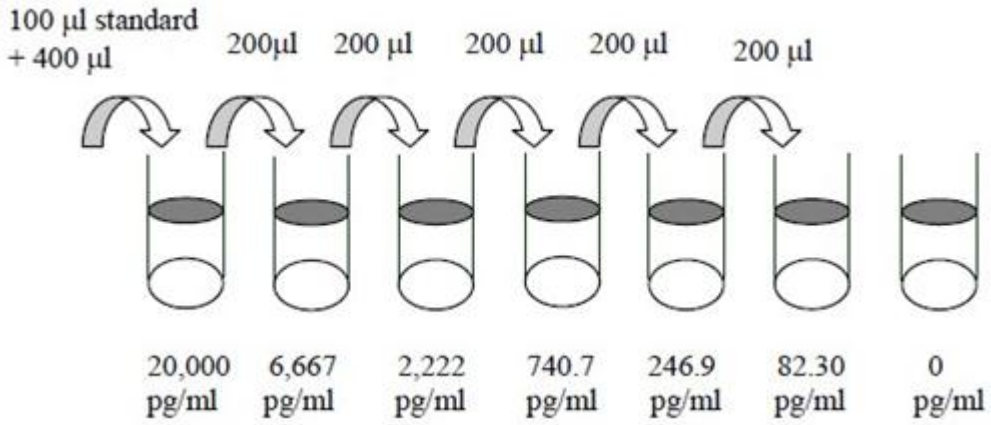


Figure 2. Dilution series for Stock standard solution (TNF- α).

Assay procedure

Both standards and samples were run in duplicate, using 96-well microliter plates.

100 µl of each usual and sample solution was added to fitting wells and coated with gelatin strip and incubated overnight with gentle shaking at room temperature for 2.5 hours or at 4 ° C. The solvent was discarded and washed 4 times with (300 µl) 1x Wash Solution using a multi-channel pipette or auto washer. Any residual wash buffer was removed after the last wash by aspirating or decanting. Plates were inverted against clean paper towels and blotted. 100 µl of 1x formulated biotinylated antibody solution was applied into each well and incubated with gentle shaking for 1 hour at room temperature. The solvent was discarded and washed 4 times using a multi-channel pipette or auto washer with (300 µl) 1x Wash Solution, as previously achieved. Any residual wash buffer was removed by aspirating or decanting after the last wash. On clean paper towels, the plate was inverted and blotted. 100 µl of prepared Streptavidin solution was applied to each well and incubated for 45 minutes at room temperature with gentle shaking. The solvent was discarded and washed 4 times using a multi-channel pipette or auto washer with (300 µl) 1x Wash Solution, as previously achieved. By aspirating or decanting after the last shower, every residual wash buffer was removed. Plates have been inverted and blotted against clean paper towels. 100 µl of tetramethyl benzidine (TMB) One-Step Substrate Reagent (Item H) was applied to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. With 50 µl of Stop Solution (Item I), each well was supplemented.

Absorbance was immediately obtained at 450nm using an ELISA micro-plate reader. For each duplicate (standards, controls and samples) set, the mean absorbance was measured.

2. Estimation of Interleukin-6 (IL-6)

For estimation of Interleukin (IL)-6 'The RayBio® Rat/Mice TNF-alpha ELISA (Enzyme-Linked Immunosorbent Assay) kit' was used.

Preparation of working Reagent

Assay Diluent C (Item L) was used for dilution of serum/plasma samples.

Assay Diluent B was diluted 5-fold with deionized or distilled water.

Preparation of standard was done as followed

670 µl Assay Diluent C (Item L) (for serum/plasma samples) in Item C (Normal Recombinant Rat/Mice IL6) was added to the vial to prepare a typical 60,000 pg/ml and mixed thoroughly. 100µl was taken into a tube with 500µl of Assay Diluent C to prepare a (10,000 pg/ml) stock standard solution. Afterwards, 200 µl of this solution was mixed into each tube with 400 µl of Assay Diluent C. Using the stock standard solution, a dilution

sequence (shown in fig 3.3) was created. Each tube was combined thoroughly prior to the next step. The zero criteria acted as Diluent C Assay. (at 0 in pg/ml).

Preparation of wash buffer
20 ml of Wash Buffer Concentrate (20x) (Item B) was diluted with deionized or distilled water to yield 400 ml of 1x Wash Buffer.

Preparation of Detection Antibody vial (Item F)
The content of detection antibody vial (item F) was proportionately added with 1x Assay Diluent C until the Detection Antibody concentration diluted 80-fold.

Preparation of HRP-Streptavidin concentrate vial (Item G)
HRP- Streptavidin concentrate was diluted to 20,000-fold with 1x Assay Diluent C.

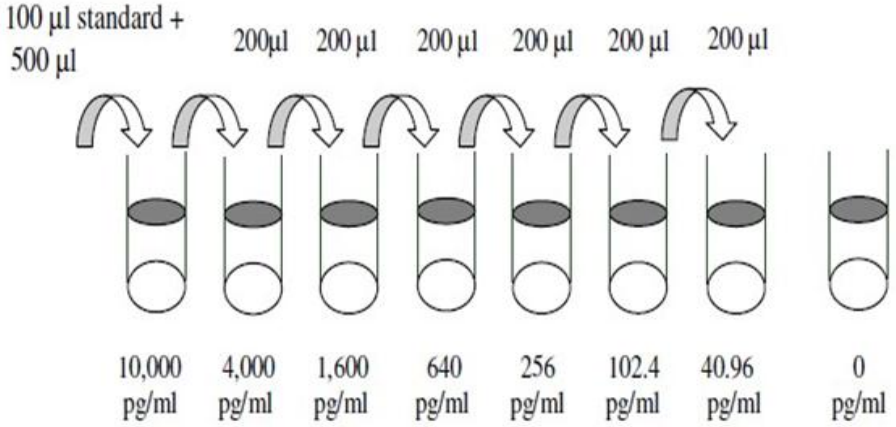


Figure 3. Dilution series for Stock standard solution (IL-6).

Assay procedure
Both standards and samples were run in duplicate using 96-well microliter panels, using
100 µl of each regular solution and sample is added to acceptable wells and coated with a gelatin strip and incubated with gentle shaking at room temperature for 2.5 hours or at 4 C overnight. The solvent was discarded and washed 4 times with (300 µl) 1x Wash Solution using a multi-channel pipette or auto washer. Any residual wash buffer was removed after the last wash by aspirating or decanting. Plates have been inverted and blotted against clean paper towels.
100 µl of 1x formulated biotinylated antibody solution was added to each well and incubated with gentle shaking for 1 hour at room temperature. The solvent was discarded and washed 4 times with (300 µl) 1x Wash Solution using a multi-channel pipette or auto washer. By aspirating or decanting after the last shower, every residual wash buffer was removed. On clean paper towels, the plate was inverted and blotted.
100 µl of prepared Streptavidin solution was applied to each well and incubated for 45 minutes at room temperature with gentle shaking. The solvent was discarded and washed 4 times with (300 µl) 1x Wash Solution using a multi-channel pipette or auto washer. By aspirating or decanting after the last shower, every residual wash buffer was removed. On clean paper towels, the plate was inverted and blotted.
100 µl of tetramethylbenzidine (TMB) One-Step Substrate Reagent (Item H) was applied to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking. There was 50 µl of stop solution (Item I) added to each well.

Absorbance was automatically obtained at 450nm using the ELISA microplate reader. Of set-in repeat (standards, controls and samples) was measured for the mean absorbance.

3. RESULTS AND DISCUSSION

Acute oral toxicity in mice was conducted according to OECD-423 recommendations. 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg were tested for toxicity. As per OECD 423 guideline up to the dosage of 2000 mg/kg protection was evaluated. The extract was considered to be healthy at all selected doses and was selected accordingly at doses up to 2000 mg/kg and no mortality was observed (Table 2). For the present investigation, 1/10th and 1/5th of 2000 mg/kg were chosen as doses. Therefore, for further research, healthy doses of 200 and 400 mg/kg of body weight were taken.

Table 2. Rate of mortality with different dose concentration.

Sr. No.	**Dose**	**No. of animal**	**Mortality***
1.	5 mg/kg	Three	0/3
2.	50 mg/kg	Three	0/3
3.	300 mg/kg	Three	0/3
4.	2000 mg/kg	Three	0/3

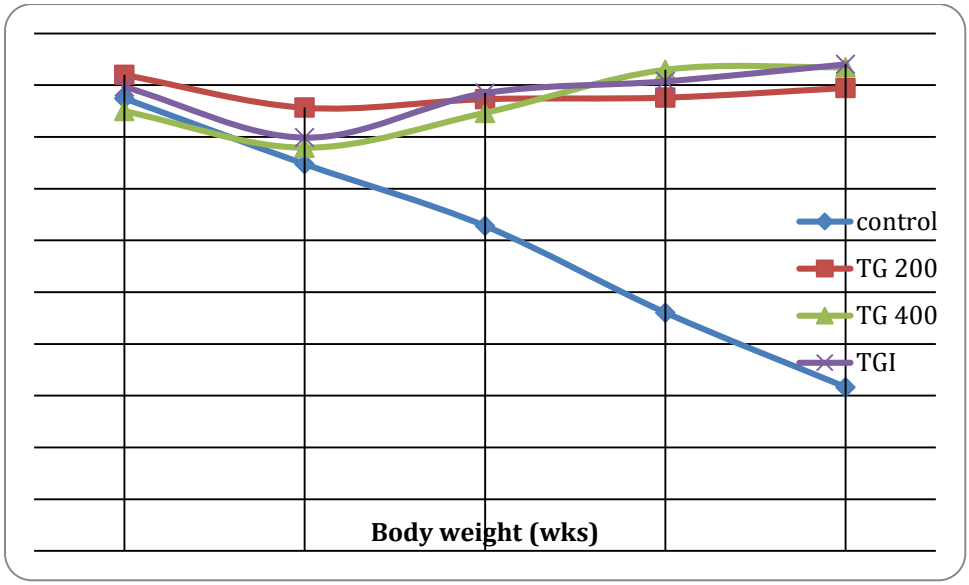
*x/3 : x animals dead in 3 test animals

In vivo Anti-cancer activity (Lung cancer)

In our research on anticancer activity, we found that tumour yield and tumour burden were not detected up to 4 weeks of daily carcinogenic compound exposure, but we observed tumour development in the 8th week and in all treated groups of mice it became maximal in the 16th week. Maximum tumour activity attributable to carcinogenicity was found in the carcinogenic control group of mice, but decreased after sample 1,2,3 and isolated compound therapy. In the isolated sample treated community of mice, the highest reduction in tumour development was found, so we concluded that samples and isolated compounds have some properties that could minimise the activity of carcinogenic compounds and reduce the formation of tumours. The various criteria, which are discussed below were studied.

Body Weight (gm)

Body weight gradually increased with time but in carcinogenic control group growth rate reduced as compared with normal animals' growth rate. Body weight increased as compared to carcinogenic group. In isolated compound group maximum body weight were seen that is direct indication of better growth rate. (Graph 1)



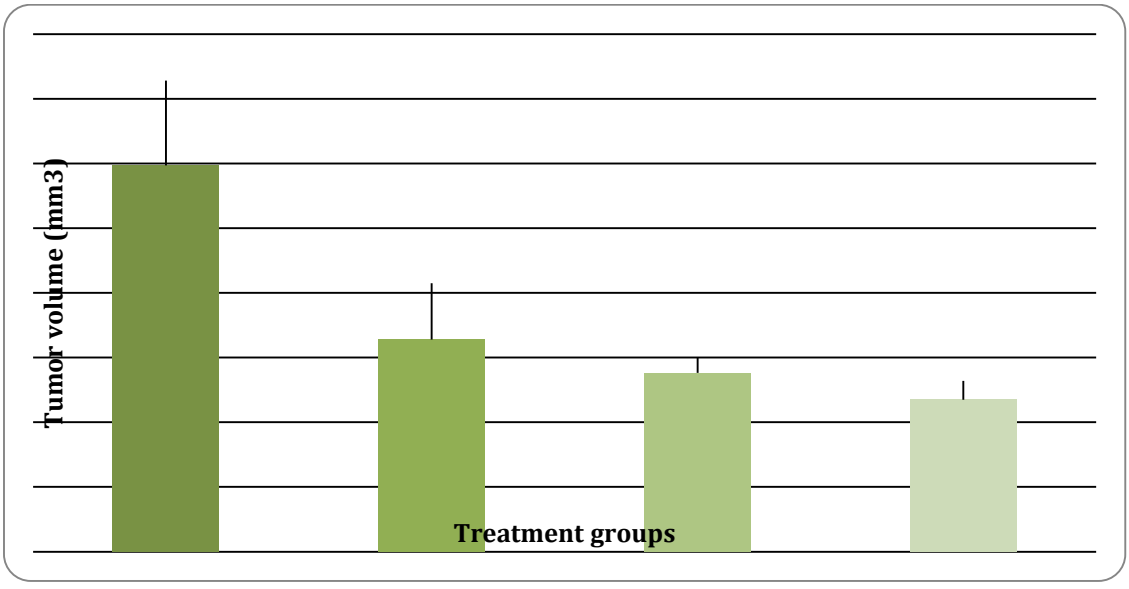
Graph 1. Body weight changes in treatment and control group of animals.

Tumor Incidence (%)

(%) Occurrence of tumours suggests instances of tumours found in natural cells of the body. We noticed in our study that the various outcomes are seen by all different classes. The number of tumour incidents in the control carcinogenic community were not detected up to 4 weeks of routine treatment of carcinogenic compounds but showed full (percent) tumour incidents at week 8 and were the same up to 16 weeks of treatment. Tumour events were decreased following treatment with Thunbergia grandiflora relative to the control group and were the same for up to 16 weeks of treatment. Compared to the extract treated classes, tumour occurrence was marginal in isolated compound therapy (percent). Therefore, we inferred from all the knowledge that the isolated compound showed optimum resistance from tumour development.

Tumor Volume

Tumor Volume of treatment groups i.e. Tumor Control, TG 200, TG 400 and TGI were represented in graphical form in Graph 2.

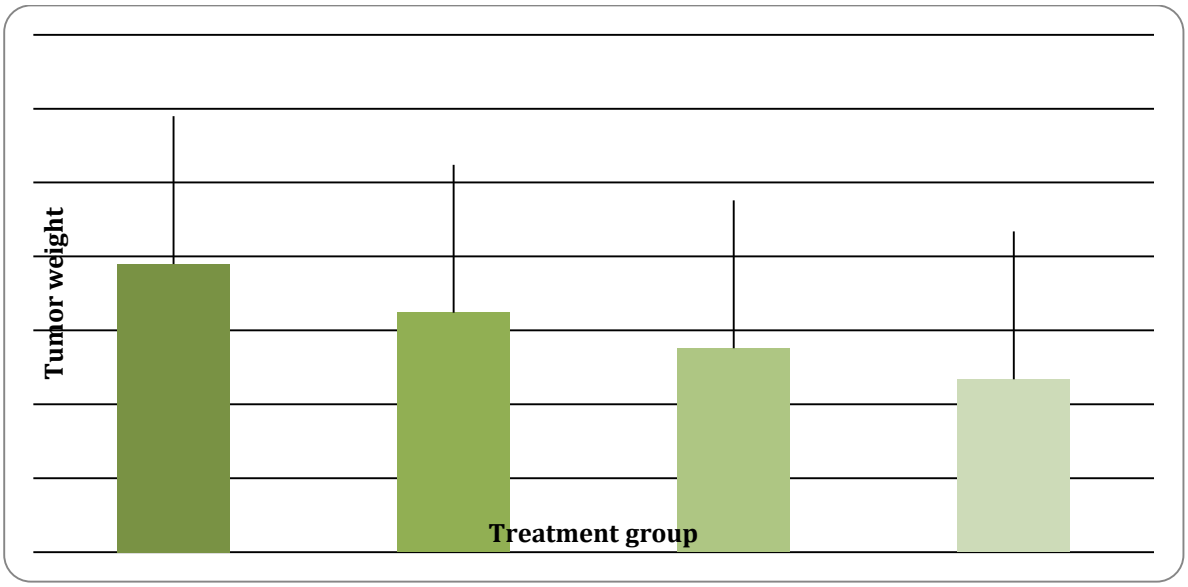


Values are expressed in Mean±SD. Statistical differences were analyzed with one-way ANOVA test. $p<0.05$

Graph 2. Tumor volume of various treatment groups.

Tumor Weight

Tumor weight of treatment groups i.e. Tumor Control, TG 200, TG 400 and TGI were represented in graphical form in Graph 3.



Graph 3. Tumor weight of various treatment groups.

Tumor Yield and Tumor Burden

For different groups tumor yield and burden are recorded in the table 3.

Table 3. Treatment group with tumor yield and tumor burden.

Treatment Group	**Tumor Yield (%)**	**Tumor Burden (%)**
Tumor Control	3.83	3.83
TG 200	2.5	2.50
TG 400	2.66	2.67
TGI	1.66	1.67

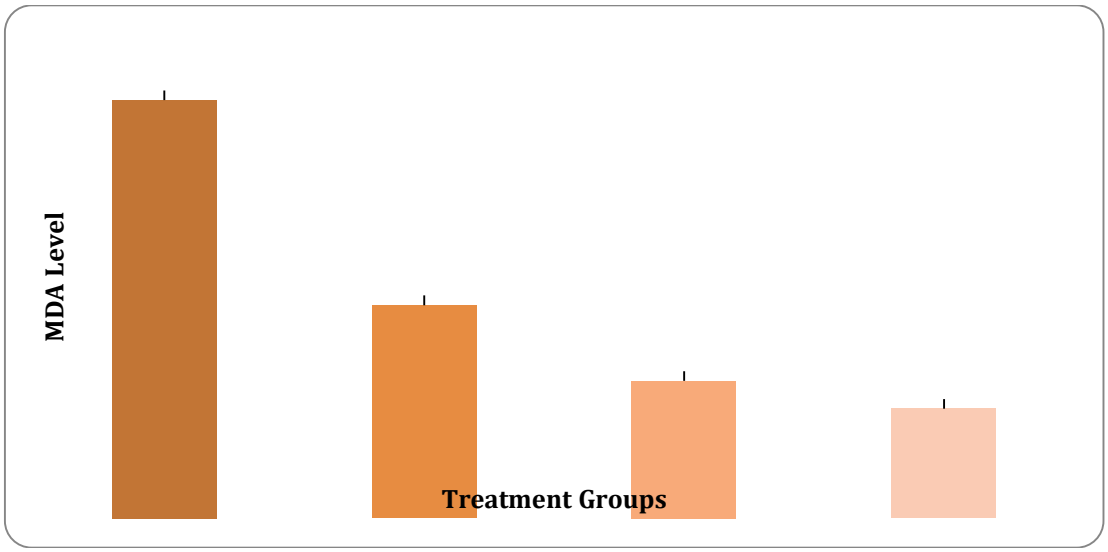
Biochemical Assay

Effect of extracts on LPO, SOD, GSH and Catalase activity in lung of carcinogen treated rats

In lung homogenates, carcinogenes increased LPO and decreased SOD, GSH, and catalase. Extracts have substantially reversed the carcinogenic impact on the stage of LPO. The isolated compound was found to be more selective for LPO, SOD, GSH and catalase than the extracts (200 mg/kg and 400 mg/kg). Tables 4, 5, 6 and 7 and Graphs 4, 5, 6 and 7 comprise the findings.

Table 4. Effect of *Thunbergia grandiflora* extract on LPO in lungs.

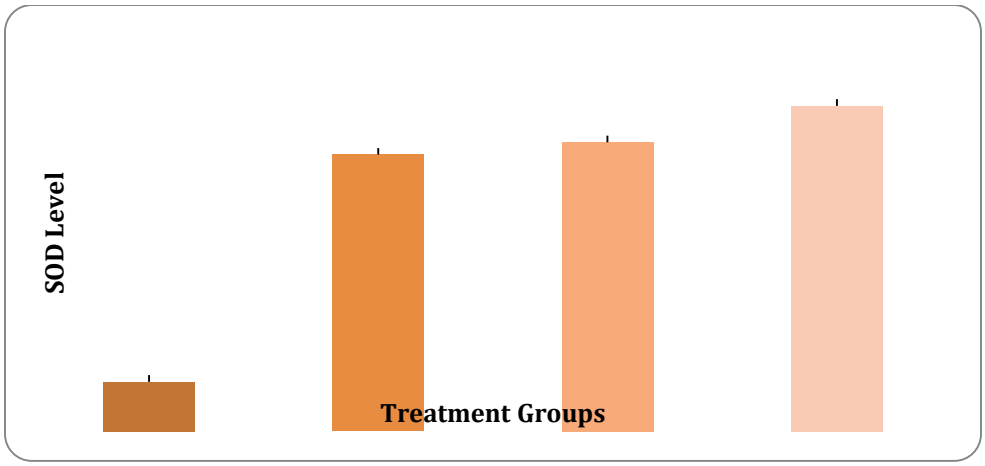
S. No.	Treatment Group	LPO (nMole/gm)
1	Tumor Control	43.25± 1.518
2	TG 200	22.03 ± 2.784
3	TG 400	14.21 ± 0.774
4	TGI	11.33 ± 0.544



All data presented in Mean±SD (N=6); P<0.05 as compared to vehicle to Control group
Graph 4. Effect of *Thunbergia grandiflora* extract on LPO in lungs.

Table 5. Effect of *Thunbergia grandiflora* extract on SOD in lungs.

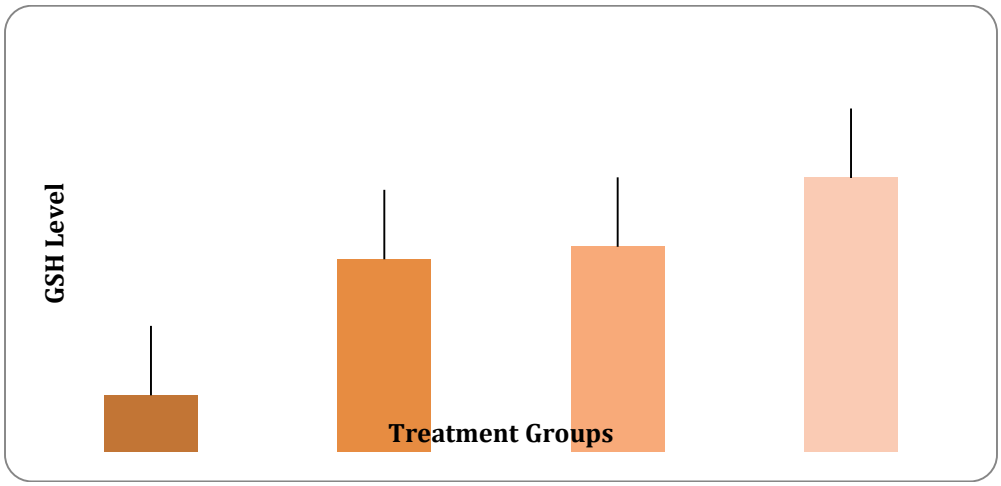
S. No.	Treatment Group	SOD (U/gm)
1	Tumor Control	7.52± 1.728
2	TG 200	41.81 ± 2.472
3	TG 400	43.69 ± 3.044
4	TGI	49.19 ± 0.818



All data presented in Mean±SD (N=6); P<0.05 as compared to vehicle to Control group
Graph 5. Effect of *Thunbergia grandiflora* extract on SOD in lung.

Table 6. Effect of *Thunbergia grandiflora* extract on GSH in lungs.

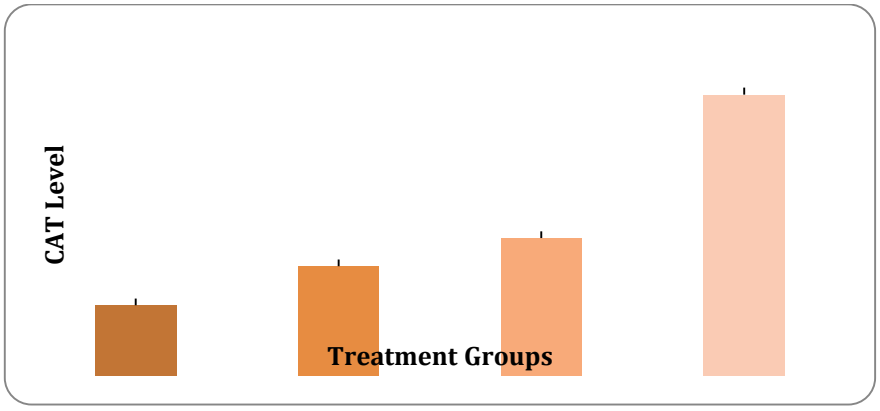
S. No.	Treatment Group	GSH (µMole/gm)
1	Tumor Control	0.814 ± 0.244
2	TG 200	2.772 ±1.064
3	TG 400	2.954 ± 1.098
4	TGI	3.943 ± 1.087



All data presented in Mean±SD (N=6); P<0.05 as compared to vehicle to Control group
Graph 6. Effect of *Thunbergia grandiflora* extract on GSH in lungs.

Table 7. Effect of *Thunbergia grandiflora* extract on Catalase in lungs.

S. No.	Treatment Group	Catalase (U/mg)
1	Tumor Control	10.22 ± 0.331
2	TG 200	15.95 ± 1.092
3	TG 400	20.06 ±1.165
4	TGI	41.09 ±8.423

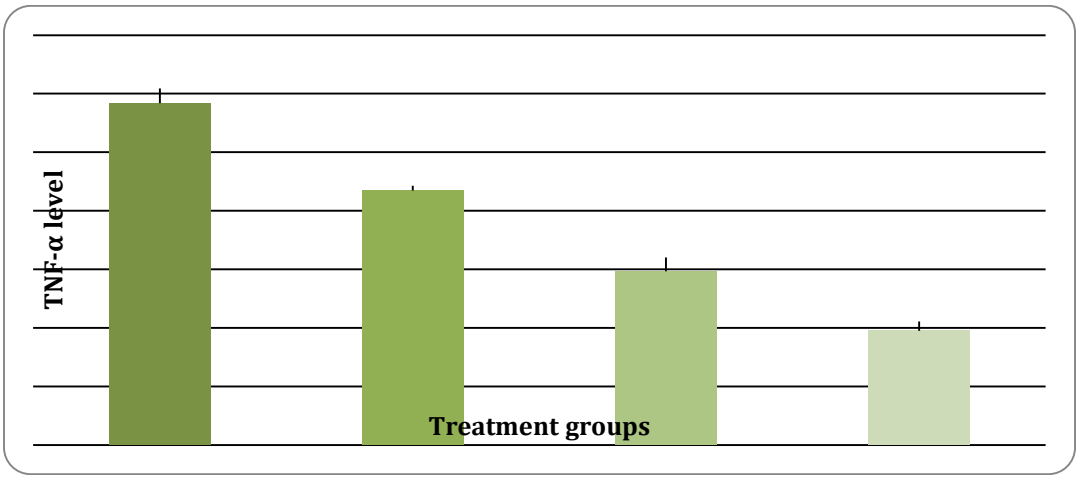


All data presented in Mean±SD (N=6); P<0.05 as compared to vehicle to Control group
Graph 7. Effect of *Thunbergia grandiflora* extract on Catalase in lungs.

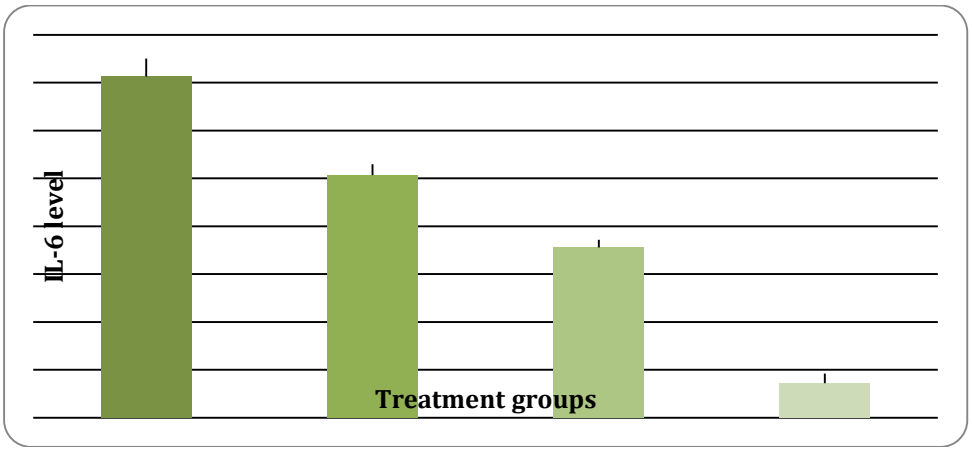
Estimation of TNF-α and IL-6

The main molecular main players in the inflammation of the cancer axis are pro-inflammatory cytokines such as TNF-a and IL-6. TNF-a plays a dual role in carcinogenesis as the symbolic inflammatory cytokine with pleiotropic functions. In the current research, a

steady rise in TNF-alpha levels after 16 weeks of therapy in animals with lung cancer could be attributed to a marked inflammatory reaction. There is also growing evidence that TNF-alpha is predominantly released by cancers and functions as an endogenous promoter of tumours. Studies have shown that administration of B(a)P facilitates the rise of TNF-alpha levels in mice carrying pulmonary tumours. At week 16 of TG 400, therapy for animal-bearing cancer greatly decreased TNF-alpha levels, and in the case of TGI, TNF-alpha levels tend to be almost similar to natural values. The IL-6 calculation also suggests the downward control of its level. The findings revealed that the administration of Thunbergia grandiflora extract to cancer-bearing mice down controlled TNF-alpha and IL-6 levels, demonstrating the immuno modulating role of Thunbergia grandiflora extract. (Chart 8 and chart 9)



All data presented in Mean±SD (N=6); P<0.05 as compared to vehicle to Control group
Graph 8. Effect of *Thunbergia grandiflora* extract on TNF- α level in lungs.



All data presented in Mean±SD (N=6); P<0.05 as compared to vehicle to Control group
Graph 9. Effect of *Thunbergia grandiflora* extract on IL-6 level in lungs.

4. CONCLUSION

Using 96-well microliter plates, all standards and samples were run in duplicate, using
In suitable wells, 100 µl of each normal solution and sample are applied and coated with a gelatin strip and incubated with gentle shaking for 2.5 hours at room temperature or overnight at 4 C. The solution was discarded and washed using a multi-channel pipette or auto washer 4 times with (300 µl) 1x Wash Solution. Any residual wash buffer was eliminated by aspirating

or decanting after the last wash. Plates have been inverted and blotted against clean paper towels.

For 1 hour at room temperature, 100 µl of 1x formulated biotinylated antibody solution was applied to each well and incubated with gentle shaking. The solution was discarded and washed using a multi-channel pipette or auto washer 4 times with (300 µl) 1x Wash Solution. Any residual wash buffer has been eliminated by aspirating or decanting after the last wash. The plate was inverted on clean paper towels and blotted.

In each well, 100 µl of prepared Streptavidin solution was added and incubated for 45 minutes with gentle shaking at room temperature. The solvent was discarded and washed 4 times using a multi-channel pipette or autowasher with (300 µl) 1x Wash Solution. Any residual wash buffer has been eliminated by aspirating or decanting after the last wash. The plate was inverted on clean paper towels and blotted.

100 µl of tetramethylbenzidine (TMB) One-Step Substrate Reagent (Item H) was applied to each well and incubated in the dark with gentle shaking for 30 minutes at room temperature. Each well had 50 µl of stop solution (Item I) added to it.

Conflict of interest

The authors declare no conflict of interest.

5. REFERENCES

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